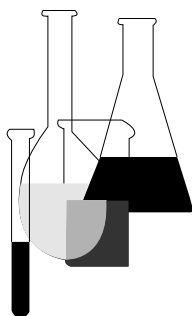




Fate, Transport and Transformation Test Guidelines

OPPTS 835.3120 Sealed-Vessel CO₂ Production Test



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

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OPPTS 835.3120 Sealed-vessel CO₂ production test.

(a) **Scope—(1) Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are the articles referenced under paragraphs (n)(2), (n)(3), and (n)(5) of this guideline.

(b) **Purpose, scope, relevance, applicability, and limits of test.** (1) This test guideline describes a method for determining ready, ultimate, aerobic biodegradability of organic chemicals by monitoring CO₂ production in sealed vessels containing the test compound and a dilute sewage inoculum. Because of the stringency of the test conditions, it can be assumed that a compound that is degraded to the extent of 60 percent or more in this test will also degrade extensively in most aerobic environmental compartments.

(2) The test method is based on the sealed vessel procedures described in the references in paragraphs (n)(2), (n)(3), and (n)(5) of this guideline, which were developed as simpler, more economical alternatives to the CO₂ production test methods described by Gledhill and Sturm (referenced in paragraphs (n)(4) and (n)(6) of this guideline). The latter two form the basis of OPPTS test guidelines 835.3100 and 835.3110 paragraph (m), respectively.

(3) Since this method provides test conditions typical of ready biodegradability tests, paragraphs (a) through (l) of OPPTS test guideline 835.3110, Ready biodegradability, are considered to be generally applicable. These paragraphs contain a glossary of key terms and extensive background information on Organization for Economic Cooperation and Development (OECD) ready biodegradability tests.

(4) This method is applicable to pure materials, including materials with very low water solubility, that can be dissolved or homogeneously dispersed in aqueous stock solutions of at least 25 mg C/L, or reproducibly introduced into test vessels as pure materials in 1–2 mg portions. This guideline is also applicable to volatile materials with Henry's law constants up to approximately 10^{-2} atm-m³/mole. However, testing of mixtures, extracts or formulated products may lead to problems in data interpretation and is not recommended.

(5) This method involves incubation of the test compound with a diluted inoculum of microorganisms from domestic secondary sewage treatment effluent in small sealed vessels for 28 days. Biodegradation is determined by monitoring CO₂ produced as the sum of gaseous CO₂ in the headspace of the vessels and the CO₂ equivalent of dissolved inorganic carbon (DIC) in the liquid phase. Alternatively, measurements can be made

on just the liquid phase after addition of alkali, or just the gaseous inorganic carbon (GIC) in the headspace following acidification of the medium. Measurements of CO₂ are made with commercial carbon analyzers, which utilize infrared detection of CO₂. Net CO₂ production provides unequivocal proof of a test compound's ultimate biodegradation, except in the unlikely event of abiotic production of CO₂.

(6) For water-soluble materials that do not adsorb to glass or biological solids, biodegradation may be confirmed by measuring disappearance of dissolved organic carbon (DOC) from the liquid phase.

(7) The simplicity of this method permits ample replicate sampling for rate determination and/or statistical evaluation of results.

(8) It may not be feasible to test materials that are toxic to the microbial population at 10 mg C/L using this method, or it may be necessary to modify the test protocol by reducing the initial concentration of test compound if instrument sensitivity permits.

(9) This method may involve hazardous materials, operations, and equipment. However, this guideline does not purport to address all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate safety and health practices. For specific safety precautions see paragraph (f) of this guideline.

(c) **Applicable ASTM standards.** Refer to the documents referenced in paragraph (n)(1) of this guideline for the following standards:

(1) D1129–90: Standard Terminology Relating to Water.

(2) D1193–91: Standard Specifications for Reagent Water (Federal Test Method and Standard No. 7916).

(3) D1293–84: Standard Test Methods for pH of Water.

(4) D2579–85: Standard Test Method for Total and Organic Carbon in Water.

(5) D2777: Standard Practice for Determination of Precision and Bias of Applicable Methods of Committee D–19 on Water.

(6) D4375–90: Standard Terminology for Basic Statistics in Committee D–19 on Water.

(7) D4839–88: Standard Test Method for Total Organic Carbon in Water by Ultraviolet, or Persulfate Oxidation or Both, and Infrared Detection.

(8) E178–80: Standard Practice for Dealing with Outlying Observations.

(d) **Summary of test method.** Biodegradation testing of organic compounds is performed by monitoring CO₂ production in small sealed vessels containing mineral nutrient medium inoculated with microorganisms from secondary sewage treatment effluent obtained from a local domestic sewage treatment plant. Vessels (160-mL gas-tight bottles are recommended) are charged with the test compound, inoculum, and mineral nutrient medium to a volume of 100 mL. The vessels are sealed with butyl rubber or neoprene septa and incubated on a gyrotory shaker at 20 °C for up to 28 days. Periodically, test vessels are sacrificed for analysis of DIC in the liquid phase and gaseous CO₂ in the headspace, using a commercial carbon analyzer. The amount of CO₂ resulting from biodegradation of the test compound is determined by comparing the total CO₂ content of the test vessels with that of controls containing no test compound. The extent of biodegradation is determined by comparing the actual amount of CO₂ produced to the maximum amount that could theoretically be produced if all of the carbon in the test compound were oxidized to CO₂.

(e) **Interpretation of test results.** (1) Interpretation of results from this test is similar to that for the Sturm test (see OPPTS 835.3110, paragraph (m)). In order to be regarded as readily biodegradable, the test compound must be degraded to the extent of at least 60 percent of theoretical CO₂ (TCO₂) production within 28 days, and this level must be attained within 10 days of the time that CO₂ production exceeds 10 percent of theoretical (“10-day window”).

(2) It is possible that the potential for meeting the 10-day window criterion may be greater with this method than with the Sturm test since in the former, all of the CO₂ produced is measured simultaneously, whereas in the latter there is a delay between the time the CO₂ is generated and the time that it is captured in the alkali CO₂ traps. For more information on this consult the reference in paragraph (n)(7) of this guideline.

(3) Like other CO₂ production test methods, this method provides unequivocal proof of ultimate biodegradation, unless CO₂ is produced from the test compound abiotically. The likelihood of this occurring is in general low, but depends on the reactivity of the test compound, the presence of reactive substrates in the test medium, and energy sources, especially solar radiation.

(4) Information on the purity of the test compound is important in interpreting test results, particularly for cases in which the results lie close to the pass level. It is emphasized that this method is recommended only for single compounds (i.e., not mixtures) of reasonable purity.

(5) Information on the microbial toxicity of the test compound or potential degradation products may be helpful in determining whether the initial concentration of test compound should be reduced, as well as in the interpretation of low or erratic CO₂ production. Such information may

be obtained from an Activated Sludge Respiration Inhibition test (OPPTS 850.6800; ISO Standard 8192), or by adding 10 mg C/L as glucose to additional test vessels containing test compound and observing the effect of the test compound on glucose biodegradation.

(6) If inhibition of test compound biodegradation due to microbial toxicity is to be avoided, it is suggested that the initial concentration of test compound be no higher than one-tenth the EC50 value (or less than the EC20 value) obtained in microbial toxicity testing. Compounds with EC50 values greater than 300 mg/L are not likely to have toxic effects in this or any other ready biodegradability test.

(f) **Safety precautions.** (1) This method involves the use of non-chlorinated sewage treatment plant effluent. Consequently, individuals performing this test may be exposed to microorganisms that are dangerous to human health. Disposable latex gloves and laboratory eyewear with splash guards should be worn during procedures involving the handling of effluent. When large volumes of effluent are being handled, for example during filtering and sparging operations (see paragraph (i)(2) of this guideline), a dust/mist respirator and laboratory footwear should also be worn.

(2) Individuals who work with sewage microorganisms may want to keep current with immunizations for polio, typhoid, hepatitis B, and tetanus.

(3) Test media should be treated with 5 percent chlorine bleach prior to disposal.

(g) **Apparatus.** (1) Test vessels. (i) Gas-tight glass vessels of 160-mL capacity (e.g., Pierce Chemical Co. 125-mL *Hypo-Vials*, or equivalent) with aluminum crimp seal caps and butyl rubber or neoprene septa.

(ii) Approximately 30 vessels per test and reference compound plus 30 additional vessels for controls are adequate for triplicate sampling at zero time and twice weekly, plus six replicate samples at day 28. However, because sample timing and replication may vary, the actual number of test vessels needed will depend upon the circumstances of the individual experiment.

(iii) New test vessels should be rinsed twice with tap water and once with deionized water, and dried in an oven at 110 °C prior to use. Vessels may be reused after thorough cleaning; for example, after cleaning at 60 °C in an ultrasonic bath, rinsing with copious volumes of distilled water and drying.

(2) Large, heavy duty gyrotory shaker. (For example, New Brunswick Scientific Model G10 equipped with universal platform, or equivalent.)

(3) Carbon analyzers. (i) For analysis of carbon in the test medium (liquid phase), the analyzer must be capable of measuring DIC and DOC in aqueous media over the range of 0 to 20 mg C/L (a suitable instrument is an OI Corporation model 700 TOC analyzer, or equivalent).

(ii) For analysis of CO₂ in headspace gas, the analyzer must be capable of measuring CO₂ over the range of 0 to 1 μg C (a suitable instrument is an Ionics model 1555b TOC analyzer with Horiba model PIR2000 NDIR CO₂ detector, or equivalent). The same analyzer, for example the Ionics 1555b, can be used for both test medium and headspace analyses, but with some loss of speed and convenience.

(4) Syringes. (i) A gas-tight, 1,000 μL cemented-needle syringe with 22° beveled, bent point, for piercing butyl rubber or neoprene septa and injecting gas-phase samples into the gas-phase analyzer (for example, Hamilton no. 1001 with no. 81317 tip, or equivalent).

(ii) A spring-loaded syringe with square end, for injecting liquid samples into Ionics-type analyzers (or equivalent), if used.

(5) Filter apparatus. Filter flask, 2- or 3-L capacity, 20-cm Büchner funnel, 18.5-cm coarse filter paper (Whatman no. 41, or equivalent), and vacuum source, for filtering sewage treatment effluent inoculum.

(6) pH meter.

(7) Flasks/Bottles. (i) Three 100-mL volumetric flasks and one 1,000-mL volumetric flask for preparing mineral nutrient medium stock solutions.

(ii) One 2,000-mL volumetric for each test compound for preparing test compound stock solutions. For water-soluble test compounds that do not precipitate in the presence of the mineral nutrient test medium, more concentrated stock solutions may be used. In this case smaller volumetric flasks are suitable.

(iii) Glass bottles or flasks, 6-L capacity, for preparation of mineral nutrient medium.

(8) Magnetic stirrers for media and sample preparation.

(9) Automated pipetting device for delivering variable volumes of liquid up to 100 mL with an accuracy of ±1 percent (for example, EM Science Optifix, or equivalent).

(10) Large laboratory oven, for drying glassware after cleaning.

(11) Ultrasonic processor (sonicator), for dispersing sparingly soluble test compounds, if needed.

(h) **Reagents.** (1) Nonchlorinated secondary effluent from an activated sludge plant treating predominantly domestic sewage should be obtained fresh on the day the experiment is initiated. Approximately 250 mL is needed for each set of 30 test vessels (refer to paragraph (g)(1)(ii) of this guideline). The undiluted effluent should contain approximately 1×10^6 microorganisms per liter.

(2) Deionized or distilled water should be free of calcium and toxic substances, especially metals such as copper. It may be desirable to saturate the water with oxygen before initiating the experiment by sparging for 20 min with clean, filtered compressed air.

(3) The following mineral nutrient medium stock solutions should be stored in the dark and discarded at the first sign of sediment, turbidity, or biological growth:

(i) Calcium chloride dihydrate: Dissolve 3.64 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 mL deionized water.

(ii) Magnesium sulfate heptahydrate: Dissolve 2.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 mL deionized water.

(iii) Ferric chloride hexahydrate and disodium ethylenediaminetetraacetic acid (EDTA): Dissolve 0.020 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.040 g Na_2EDTA in 100 mL deionized water.

(iv) Monobasic potassium phosphate, dibasic potassium phosphate, dibasic sodium phosphate heptahydrate, and ammonium chloride: Dissolve 8.50 g KH_2PO_4 , 21.75 g K_2HPO_4 , 50.30 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 0.50 g NH_4Cl in 1 L of deionized water.

(4) Test compound. (i) Test compound stock solution or stable dispersion is normally prepared to yield 25 mg C/L as test compound in deionized or distilled water. Two liters of this stock solution or stable dispersion is sufficient to dose 30 test vessels (refer to paragraph (g)(1)(ii) of this guideline). More concentrated stock solutions may be prepared for soluble test compounds that do not precipitate in the presence of mineral nutrient medium. If necessary, the pH of the stock solution or stable dispersion may be adjusted with HCl or NaOH to 7.2 ± 0.2 , provided that no precipitation or reaction of the test compound occurs.

(ii) For some test compounds, such as insoluble liquids, it may be preferable to add the compound directly to test vessels using a microliter syringe. Alternatively, very viscous compounds may be spread on a tared coverslip which is then placed in the test vessel.

(iii) Test compounds known to be toxic to sludge microorganisms at 10 mg C/L may be tested at lower initial concentrations down to a minimum level of 2 to 5 mg C/L, the actual value depending on the sensitivity of the carbon analyzer.

(5) Reference compound. (i) In order to check the activity of the inoculum, one or more reference compounds that meet the criteria for ready biodegradability are tested by setting up a series of test vessels parallel to those with test compound.

(ii) Suitable compounds are aniline (freshly distilled) and phthalic or trimellitic acid, but sodium acetate, dextrose (glucose), and sodium benzoate may be too biodegradable to be useful for this purpose.

(iii) For test compounds with very low or negligible water solubility, it may be desirable to employ reference compounds of similar solubility—for example, sodium stearate.

(6) CO₂-free compressed air or nitrogen for sparging the inoculum to ensure that it is free of dissolved CO₂. The delivery line should be equipped with a large gas diffusing stone (Fisher no. 11–139A or equivalent) for maximum sparging efficiency.

(7) Calibration gas for headspace analysis should be of certified standard grade and contain no more than approximately 0.25 percent CO₂ by volume, with the balance being nitrogen.

(8) A solution of NaHCO₃ in the range of 0 to 20 mg C/L as total inorganic carbon (TIC) is suitable for use as a calibration solution for liquid-phase DIC analysis.

(9) A solution of potassium hydrogen phthalate in the range of 0 to 20 mg C/L as total organic carbon (TOC) is suitable for use as a calibration solution for liquid-phase DOC analysis.

(i) **Procedure.** (1) Prepare test and reference compound stock solutions the day before the test is to begin, dilute in triplicate, and analyze for test and reference compound concentrations and homogeneity using organic carbon analysis or other appropriate method.

(2) On the day the test is initiated, use a Büchner funnel to filter freshly collected secondary sewage treatment effluent to remove particulates and begin sparging the filtered effluent with CO₂-free air or nitrogen to remove CO₂ and dissolved carbonates and bicarbonates. Interrupt the sparging process approximately every 15 min to monitor effluent TIC, TOC and pH. If necessary, add sufficient 1N HCl to reduce the pH to 6.5. Repeat this procedure until the DIC is less than 5 mg C/L. DOC should not exceed 10 mg C/L.

(3) Inoculated mineral nutrient medium. (i) Prepare inoculated mineral nutrient medium by mixing ingredients listed in the following Table 1. in a 6-L glass bottle or flask:

Table 1.—Inoculated mineral nutrient medium

Constituent	Volume (mL)
Deionized or distilled water	5,370
Calcium chloride stock solution	10
Magnesium sulfate stock solution	10
Ferric chloride stock solution	10
Phosphate buffer stock solution	100
Inoculum (CO ₂ -free, pH 6.3)	500
Total volume	6,000

(ii) Six liters of inoculated medium is sufficient for 99 test vessels. Approximately 30 vessels are needed for each test and reference compound.

(iii) The actual volume of inoculum needed may vary from 50 to 1,000 mL depending upon the microbial density. Undiluted inoculum should contain approximately 1×10^6 microorganisms per liter, but the actual microbial density may have to be determined by the investigator. Adjust the volume of deionized or distilled water as necessary to achieve a final volume of 6 L.

(4) Dispense 60 mL of inoculated mineral nutrient medium into each test vessel.

(5) Dispense 40 mL of test or reference compound stock solution containing 25 mg C/L into each test vessel in the appropriate series of vessels. If a more concentrated stock solution is used, bring the total volume in each vessel to 100 mL with deionized or distilled water. For controls without test or reference compound, replace test/reference compound stock solution with deionized or distilled water.

(6) Concentrations of all components of the test system at the initiation of the test are as given in the following Table 2.:

Table 2. Concentration Requirements for Test System

pH of Inoculated Test Medium = 7.2 ± 0.2

Component	Concentration (mg/L unless otherwise noted)
Calcium chloride dihydrate—CaCl ₂ ·2H ₂ O	36.4
Magnesium sulfate heptahydrate—MgSO ₄ ·7H ₂ O	22.5
Ferric chloride hexahydrate—FeCl ₃ ·6H ₂ O	0.2
EDTA disodium salt—Na ₂ EDTA	0.4
Potassium phosphate, monobasic—KH ₂ PO ₄	85.0
Potassium phosphate, dibasic—K ₂ HPO ₄	217.5
Disodium phosphate, heptahydrate—Na ₂ HPO ₄ ·7H ₂ O	503.0
Ammonium chloride—NH ₄ Cl	5.0
Inoculum (filtered effluent)	(5 % by volume)
Organic carbon from test /reference compound	10.0

(7) Seal test vessels immediately with neoprene or butyl rubber septa and aluminum crimp seal caps. Set aside an appropriate number of vessels (typically three) from each series (test compound/reference compound/control) for zero time analysis.

(8) Pack the remaining vessels in boxes, cover tightly to prevent light from striking the vessels, and place the boxes on a gyrotory shaker. Incubate at 20 ± 1 °C (68 °F) with shaking at approximately 150 rpm.

(j) **Sampling and analysis.** (1) Calibrate the carbon analyzer to be used for headspace analysis using calibration gas.

(2) Calibrate the carbon analyzer to be used for analysis of the liquid phase using standard solutions of sodium hydrogen carbonate for inorganic carbon and potassium hydrogen phthalate for organic carbon.

(3) At zero time, withdraw a sample of headspace gas from each test vessel designated for sacrifice at zero time. Use a gas-tight syringe and needle to pierce the butyl rubber or neoprene septum and collect the sample, and inject the sample into the carbon analyzer to analyze for CO₂. Test compound/reference compound/control vessels are normally sacrificed in triplicate at zero and subsequent times.

(4) Remove the septum from the same vessel and use a syringe to collect a sample of liquid for analysis. Collect the sample immediately after removing the septum. Measure DIC by injecting the sample into the appropriate carbon analyzer. If desired, analyze for DOC also.

(5) Alternatively, measurements can be made on just the liquid phase after addition of 1 mL of 10 N NaOH to the medium, or just the headspace following acidification of the medium with 1 mL of 10 N HCl. Of the two methods alkali addition may be preferable, since a small leak in the test vessel could lead to significant loss of headspace CO₂ following acidification. If either of these options is selected, the acid or alkali should be added before the septum is removed. If only headspace is to be analyzed, headspace gas should also be sampled before the septum is removed. Allow 1 h between adding acid or alkali and sampling.

(6) After the experiment is initiated, remove test vessels from the shaker and analyze according to a time schedule designed to match the degradation rate of the test compound. Twice weekly sampling is often sufficient to establish a smooth curve depicting cumulative CO₂ generated vs time.

(7) On day 28 of the experiment remove six vessels from each series and analyze as above. Alternatively, the experiment may be terminated before 28 days if the test compound biodegradation curve reaches a plateau and does not change significantly over three or more sampling times; and it may be prolonged beyond 28 days if the degradation curve shows that

degradation has started, but that a plateau has not been reached by day 28. Analysis of six rather than three replicates from each series on the last day of the experiment permits more accurate assessment of the final extent of biodegradation.

(k) **Calculations.** (1) The amount of carbon as evolved CO₂ appearing in the liquid phase (i.e., dissolved inorganic carbon) is calculated as follows:

$$C_w = V_w [(sample\ DIC_t - control\ DIC_t) - (sample\ DIC_0 - control\ DIC_0)]$$

where

V_w = volume of liquid phase (mL) (normally 100mL)

C_w = inorganic carbon in liquid phase (μg)

sample DIC_t = sample DIC at time t (μg/mL)

control DIC_t = control DIC at time t (μg/mL) (mean of all replicates)

sample DIC₀ = sample DIC at zero time (μg/mL)

control DIC₀ = control DIC at zero time (μg/mL) (mean of all replicates)

(2) The amount of carbon as evolved CO₂ appearing in the gas phase is calculated as follows:

$$C_g = V_g [sample\ GIC_t - control\ GIC_t]$$

where

V_g = volume of headspace (mL) (normally 60 mL)

C_g = inorganic carbon in headspace (μg)

sample GIC_t = sample GIC at time t (μg/mL)

control GIC_t = control GIC at time t (μg/mL) (mean of all replicates)

(3) Total observed CO₂ production as a percentage of theoretical (% TCO₂) is calculated as follows:

$$\% TCO_2 = [(C_w + C_g)/(TOC_0 \times V_w)] \times 100$$

where

TOC₀ = Test or reference compound TOC in solution at zero time (μg C/mL)

C_w , C_g , V_w are defined under paragraph (k)(1) of this guideline. TOC₀ may be obtained either by measurement or calculation.

(4) The sample standard deviation for percent TCO₂ and 95 percent confidence limits for mean percent TCO₂ at any sampling time are calculated for each set of three replicates (test or reference compound) as follows:

$$s = \sqrt{\sum_i (x_i - \bar{x})^2 / n - 1}$$

95 percent confidence limits of mean percent TCO₂ =

$$\bar{x} \pm s \cdot t / \sqrt{n}$$

where

$$\bar{x} = \text{mean \%TCO}_2$$

x = percent TCO₂

s = sample standard deviation for percent TCO₂

n = number of replicates (normally three)

t = value of Student's t (two-tailed test) at (n – 1) degrees of freedom and 0.05 significance level (available from standard statistical tables)

(1) **Quality assurance.** To assure the integrity of data developed using this method and to comply with current regulatory requirements, a quality assurance program meeting EPA, FDA (United States Food and Drug Administration), or OECD guidelines should be followed.

(m) **Documentation.** (1) A study plan giving a general overview of the study goals and procedures should be prepared before the study is initiated. If a substantive modification of this method is deemed necessary for the test compound, such a modification should be documented in the study plan.

(2) Results of the study should be documented in a final report. The final report should include the following:

(i) Names of study, investigators, and laboratory.

(ii) A brief description of the test compound including its identifying number in the logbook, chemical names, composition, and other appropriate information.

(iii) Summary of the test method including deviations from the written method.

(iv) A brief description of any supplementary tests performed, such as microbial toxicity tests or analyses to verify test compound concentration and/or homogeneity, and the results of those tests.

(v) Tabular and graphical presentations of percent TCO₂ as a function of time after test initiation. The final extent of degradation should be calculated from the six replicates for each test and reference compound that are sampled at the termination of the test, and expressed as the mean percent TCO₂, sample standard deviation, and 95 percent confidence limits for the mean. The report should state whether or not the test compound met the 10-day window criterion.

(vi) A list of relevant references including all notebook pages and computer files containing raw data from the study.

(n) **References.** The following references should be consulted for additional background material on this test guideline.

(1) American Society for Testing and Materials (ASTM). Annual Book of ASTM Standards, Volumes 11.01 and 11.02 on Water and Environmental Technology, and Volume 14.02 on General Methods and Instrumentation (1993).

(2) Birch, R.R. and R.J. Fletcher. The application of dissolved inorganic carbon measurements to the study of aerobic biodegradability. *Chemosphere* 23:507–524 (1991).

(3) Boatman, R.J. et al. A method for measuring the biodegradation of organic chemicals. *Environmental Toxicology and Chemistry* 5:233–243 (1986).

(4) Gledhill, W.E. Screening test for assessment of ultimate biodegradability: LAS. *Applied Microbiology* 30:922–929 (1975).

(5) Struijs, J. and J. Stoltenkamp. Headspace determination of evolved carbon dioxide in a biodegradability screening test. *Ecotoxicology and Environmental Safety* 19:204–211 (1990).

(6) Sturm, R.N. Biodegradability of nonionic surfactants: Screening test for predicting rate and ultimate biodegradation. *Journal of the American Oil Chemists' Society* 50:159–167 (1973).

(7) Weytjens, D. et al. The recovery of carbon dioxide in the Sturm test for ready biodegradability. *Chemosphere* 28:801–812 (1994).